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A cyclic AMP-responsive element-binding transcriptional activator in Drosophila melanogaster, dCREB-A, is a member of the leucine zipper family.
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A Cyclic AMP-Responsive Element-Binding Transcriptional Activator in *Drosophila melanogaster*, dCREB-A, Is a Member of the Leucine Zipper Family

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In this report, we describe the isolation and initial characterization of a *Drosophila* protein, dCREB-A, that can bind the somatostatin cyclic AMP (cAMP)-responsive element and is capable of activating transcription in cell culture. Sequence analysis demonstrates that this protein is a member of the leucine zipper family of transcription factors. dCREB-A is unusual in that it contains six hydrophobic residue iterations in the zipper domain rather than the four or five commonly found in this group of proteins. The DNA-binding domain is more closely related to mammalian CREB than to the AP-1 factors in both sequence homology and specificity of cAMP-responsive element binding. In embryos, dCREB-A is expressed in the developing salivary gland. A more complex pattern of expression is detected in the adult; transcripts are found in the brain and optic lobe cell bodies, salivary gland, and midgut epithelial cells of the cardia. In females, dCREB-A is expressed in the ovarian columnar follicle cells, and in males, dCREB-A RNA is seen in the seminal vesicle, ejaculatory duct, and ejaculatory bulb. These results suggest that the dCREB-A transcription factor may be involved in fertility and neurological functions.

In a variety of systems, it has been shown that growth factors, hormones, and neurotransmitters regulate changes in cell morphology, differentiation, and intercellular communication through second messenger-coupled cell surface receptors. The signaling cascades stimulated by these receptors ultimately result in the activation of specific transcription factors that induce discrete batteries of genes. Identification of these transcription factors and their target enhancers is a key issue in understanding the mechanisms by which signal transduction pathways stimulate long-term changes in cellular function.

One of these enhancers, the cyclic AMP (cAMP)-responsive element (CRE), was originally described as an 8-bp sequence, 5′-TGACGTCA-3′, in the somatostatin gene (36). Related sequences have been identified near the promoters of many other cellular genes, where they appear to contribute to aspects of basal, regulated, and tissue-specific expression (3, 41). CRE sequences have also been identified in viral promoters, including those in the adenoviral early genes, where they appear to be involved in the transcriptional regulation by E1A, and in the human T-cell lymphotropic virus type I (HTLV I) long terminal repeat, where they participate in the regulation by $p40_{tax}$ (61). In some instances, these viral sequences are also capable of conferring cAMP-mediated transcriptional stimulation (46). Although the CGTCA motif appears to be the most invariant feature of a "classical" CRE (13), the arrangement of CRE motifs and the composition of flanking sequences appear to be important in defining the functions of the enhancer in specific genes. The question of how the CRE can mediate so many different modes of regulation, however, remains unanswered.

Using DNA-affinity chromatography, Montminy and Bilezikjian (35) purified the CRE-binding protein CREB from PC12 cells, a rat pheochromocytoma cell line. The cDNA cloning of human CREB indicated that it is a member of the bZip family of transcription factors, containing a basic DNA-binding domain adjacent to a leucine zipper motif (24). The amino-terminal activation domain contains consensus phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), glycogen synthase kinase-3, calcium-calmodulin (CaM) kinase II and casein kinase II (17), suggesting that CREB can mediate the effects of multiple signal transduction pathways. The data of Sheng et al. (50, 51) suggest, for example, that CREB can be activated through the Ca^{2+}-calmodulin-dependent kinases.

The diversity of CRE actions could also result from the expression of distinct CREB genes, alternately spliced CREB mRNA precursors, or posttranslational modifications of CREB proteins. Recent studies have shown that each of these mechanisms contributes to different aspects of CRE regulation. For example, Maekawa et al. (32) identified a protein, designated CRE-BP1, that contains a DNA-binding region related to that of CREB. This protein is larger than CREB and contains a distinct activation domain. CRE-BP1 retains consensus sites for PKA and PKC, but regulation through these pathways has not been demonstrated. The dimerization domain of CRE-BP1 is related to the corresponding region in c-*jun*, and it has been shown that CRE-BP1 dimerizes with c-*jun* but not CREB (27). Interestingly, association with CRE-BP1 changes c-*jun* from a phorbol ester response element (TRE)-binding protein to a CRE-binding protein. Hai et al. (21) have cloned several factors from HeLa cells that bind to the activating transcription factor (ATF) site, an adenovirus enhancer that is closely related to the somatostatin CRE. One of these factors, ATF-1, is highly homologous to CREB but is the product of a different gene. Rehfuss et al. (42) have recently shown that

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this factor, like CREB, is regulated by PKA. Another CRE-binding protein, designated CREM, has been shown to down-regulate cAMP-induced transcription (15). To date, at least 10 different mammalian CREB homologs have been identified (18), many capable of generating multiple alternatively spliced isoforms (59).

Given the number of mammalian CRE-binding proteins, characterizing the functional contributions of each of these proteins to specific aspects of second messenger-regulated gene transcription is a challenging task. Our goal was to determine whether homologs of CREB existed in a simpler eukaryotic system that could be analyzed by genetic manipulations. Ultimately, by identifying and mutating these factors in such a system, we may be able to assign particular functions to individual factors and determine the functional significance of different heterodimer combinations. We chose to use *Drosophila melanogaster* for these studies because genes homologous to those involved in mammalian signal transduction pathways have been identified in this organism and mutations in several of these genes have been found to result in specific developmental defects (2, 8, 9, 19, 29, 31, 52, 56). Additionally, the existence of *Drosophila* homologs for c-*jun* and c-*fos* (39) suggested that CREB-like transcription factors might be found in this species. In view of the diversity of mammalian CRE-binding proteins, we anticipated that some of the *Drosophila* CREB proteins would respond to cAMP and others might respond to different signaling pathways. Unlike the situation in mammalian systems, *Drosophila* offers genetic approaches to sorting out the biological functions of these non-cAMP-responsive factors.

In this paper, we describe the isolation and initial characterization of a *Drosophila* protein, which we have designated dCREB-A, that is homologous to mammalian CREB. This protein binds to the CRE sequence and belongs to the leucine zipper family of transcription factors. In cell culture, dCREB-A can act as a transcriptional activator. Although this *Drosophila* protein does not contain a PKA phosphorylation site, it does contain three CaM kinase II sites, suggesting that it may stimulate transcription through the CaM kinase II pathway.

MATERIALS AND METHODS

Isolation of the dCREB-A cDNAs. Oligonucleotides homologous to the somatostatin CRE containing flanking *Bam*HI sites (5′-GATCCTTGGCTGACGTCAGAGAGAGA-3′) were annealed and labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Tandem repeats were then generated by using T4 DNA ligase. These ligated probes were used to screen a λgt11 cDNA expression library made from 0- to 24-h-old *Drosophila* embryo mRNA (kindly provided by John Tamkun). Approximately 600,000 plaques, plated at 30,000 plaques per plate (150 by 15 mm), were screened by the method of McKnight (33) for recombinant clones expressing CRE-binding proteins. A single cDNA was isolated in this manner, which was then labeled with [α-^{32}P]dCTP and used to screen 4- to 8-h-old and 12- to 24-h-old embryonic plasmid cDNA libraries (kindly provided by Nick Brown [7]). Among the 14 cDNAs isolated was a full-length 4.478-kb clone which was sequenced and used to generate the proteins and probes described below. Both the original λgt11 clone and the full-length clone isolated from the plasmid cDNA library were labeled and hybridized to polytene chromosomes as described by Johnson-Schlitz (28).

DNA sequencing. The 4.478-kb dCREB-A cDNA was subcloned as two *Eco*RI-*Hin*dIII fragments into pBluescript-KS+ (Stratagene). Additional restriction fragments were subcloned into pBluescript-KS+, PBCKS+ (Stratagene), or pBS+/− (Stratagene). The fragments were sequenced as double-stranded DNA with α-^{35}S-deoxynucleoside triphosphates, and the kit was provided by Pharmacia, according to their protocol, except that de-aza dGTP instead of dGTP was used. Nested exo III deletions were made for the regions that could not be covered by overlapping restriction fragments. The sequence of the dCREB-A coding region presented in Fig. 1 is the complete sequence from both strands of the cDNA. The sequence of the 5′ and 3′ untranslated regions is from a single strand of the cDNA.

***In situ* hybridizations.** dCREB-A sense and antisense single-stranded DNAs were generated with digoxigenin-11-dUTP (Boehringer Mannheim) by the polymerase chain reaction and were reduced to small fragments by being boiled for 40 min. Whole-mount embryos were probed as described by Tautz and Pfeile (57) and Fleming et al. (14). The specificity of the dCREB-A expression pattern was controlled by hybridizing with the probes from the sense strand.

To analyze the expression pattern in adults, dCREB-A sense and antisense RNA probes were labeled with ^{35}S-UTP by using the Pharmacia TransProbe T kit and following their protocol. The probes were reduced to 100-bp fragments by being heated to 60°C for 50 min in 0.1 M carbonate buffer (pH 10.2). Whole flies were frozen in O.C.T. and sectioned with a cryostat microtome (Leitz, Wetzlar, Germany) at −18°C. The sections were mounted on gelatin-subbed slides and processed immediately by the method described by Hafen and Levine (20), except that the hybridization buffer and first wash were made 10 mM in dithiothreitol and the slides were treated with 20 μg of RNase A per ml in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min after the first wash. Labeling within the head sections was lost with RNase treatment; therefore, this step was omitted for this series.

dCREB-A gel shift analysis. The dCREB-A *Nco*I-*Bgl*II fragment containing the entire coding sequence was subcloned into the pET-11d (Novagene) expression vector. The dCREB-A protein was expressed in a BL21(DE3)pLysE host and isolated by the protocol for expression and purification of glutathione S transferase fusion proteins described by Smith and Corcoran (53). The extract was diluted 1:100 into phosphate-buffered saline–10% glycerol, and for each reaction mixture, 1 μl was added to a 20-μl reaction buffer [50 mM Tris (pH 8.0), 50 mM NaCl, 50 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, 5 mM spermidine, 0.05% Nonidet P-40, 3 μg of bovine serum albumin per reaction mixture, and 1 μg of poly(dI-dC) per reaction mixture]. This mixture was incubated for 15 min at room temperature. The CRE oligonucleotide was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and kept at a concentration of 10 pmol/100 μl. One microliter of the labeled CRE (0.1 pmol) was added to each reaction mixture, and the incubation was allowed to continue for an additional 15 min. The reaction mixtures were loaded onto a 4% native polyacrylamide gel and run at 10°C in 0.5× Tris-borate-EDTA. For the competition experiments, various concentrations of unlabeled oligonucleotide were added to the first incubation mixture.

dCREB-A transfection assays. A Rous sarcoma virus (RSV) vector expressing a dCREB-A-GAL4 fusion protein was constructed by subcloning the *Nco*I-*Bst*EII fragment containing the dCREB-A activation domain into the *Xba*I

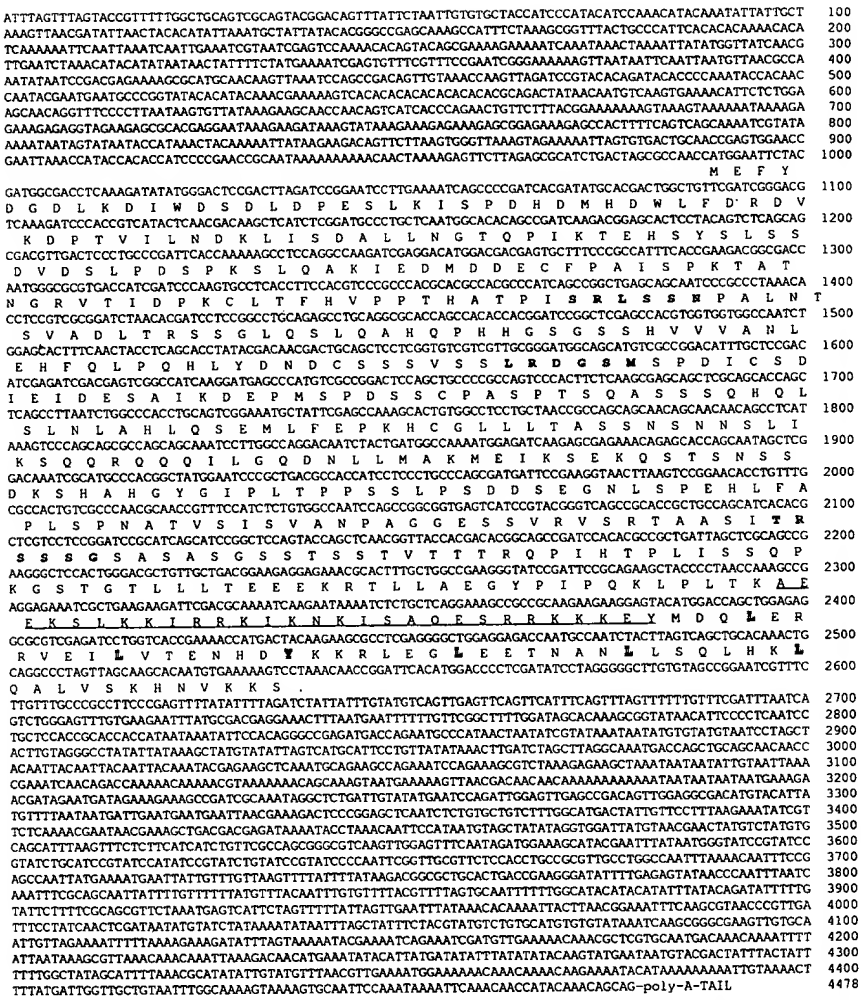

FIG. 1. DNA and amino acid sequence of dCREB-A. The six hydrophobic residues of the leucine zipper are emphasized, the basic DNA-binding domain is underlined, and the consensus CaM kinase II sites are in boldface type. The consensus CaM kinase II site is XRXXS*X.

site of pRSV-GAL. This vector, kindly provided by Michael Gilman, contains the GAL4 DNA-binding domain (1–147). The proper fusion was confirmed by sequence analysis. The GAL4-chloramphenicol acetyltransferase (CAT) reporter was the GAL4/E1bTATA plasmid (30), also provided by Michael Gilman. JEG-3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and were plated at a density of 3.5×10^5 cells per plate 18 h prior to transfection. Transfections were carried out as described elsewhere (10), in the following amounts: 5 μg of GAL4-CAT, 2 μg of RSV-luciferase, and 10 μg of dCREB-A-GAL4 or Rc-RSV (Invitrogen) as a control. Bluescript DNA was used to bring the total amount of DNA per plate to 37 μg. The cells were washed, and fresh medium was added after 24 h. To test the response of dCREB-A to cAMP, half of the plates were treated with 0.2 mM 8-(4-chlorophenylthio)-cAMP. The cells were collected after an additional 24 h, and the CAT and luciferase activities were determined as described elsewhere (12, 49). CAT activity was normalized to the luciferase activity as a control for transfection efficiency.

Nucleotide sequence accession number. The dCREB-A sequence has been assigned GenBank accession number M87038.

RESULTS

Isolation of *Drosophila* CREB cDNAs. Data base analysis indicated that a sequence homologous to that of the mammalian CRE was conserved in several *Drosophila* genes. Our studies focused on identifying *Drosophila* transcription factors that were capable of binding to this element. Initially, we attempted to isolate genes encoding these transcription factors by screening *Drosophila* libraries at low stringency with radioactive probes derived from the mammalian CREB cDNA. These screens were unsuccessful, presumably because of nucleotide divergence of regions that are relatively conserved at the protein level. We then screened an expression library made from 0- to 24-h-old *Drosophila* embryos for clones that could produce CRE-binding proteins and identified a single cDNA among the 600,000 recombinant plaques screened. The 1.136-kb cDNA fragment was subcloned, sequenced, and then used as a probe to screen additional cDNA libraries made from embryos at between 4 to 8 and 12 to 24 h of development. One 4.478-kb clone was identified among the 14 cDNA clones isolated in these screens, and this cDNA was subcloned and sequenced. The sequence for the *Drosophila* CRE-binding protein, designated dCREB-A, is given in Fig. 1.

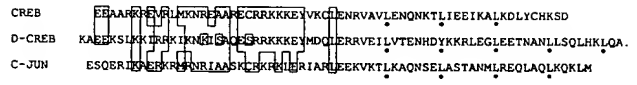

FIG. 2. Comparison of the leucine zippers and DNA-binding domains in dCREB-A, CREB, and c-*jun*. Shaded amino acids in the DNA-binding domain are identical. Boxed leucine is the first residue of the leucine zipper region. The black dots identify the other leucines and third-position tyrosine of the zipper.

The cDNA consists of 988 bp of 5′ untranslated sequence, 1.548 kb of open reading frame that encodes a 516-amino-acid protein with a calculated molecular mass of 56.3 kDa, and 1.942 kb of 3′ untranslated sequence. Like the mammalian CREB proteins, dCREB-A has a carboxy-terminal leucine zipper, an adjacent, highly basic DNA-binding domain, and an amino-terminal transcriptional-activation domain. The binding domain shows 48% homology with the corresponding region in mammalian CREB and includes the sequence RRKKKEY abutting the leucine zipper domain, which is exactly conserved among CREB, CREM, and ATF-1 (40). The leucine zipper of dCREB-A is unusual in that it has a tyrosine substitution at the third leucine position. Nucleotides encoding this tyrosine were present in multiple cDNA clones that were isolated from separate libraries. Another unusual feature is the length of the leucine zipper. While the CREB zippers are defined by four leucine iterations, the dCREB-A zipper consists of six hydrophobic residue iterations. The binding domains and leucine zippers of dCREB-A, CREB, and c-*jun* are compared in Fig. 2.

Mammalian CREB is phosphorylated in response to cAMP on a consensus PKA site within the acidic activation region. The dCREB-A protein does not contain a typical PKA site within its activation domain but does contain three possible CaM kinase II sites (Fig. 1).

The dCREB-A clones were used to map the dCREB-A gene to position 71CD on the left arm of chromosome 3 (data not shown). This region lies in an ecdysone-inducible puff and contains several well-characterized genes, including gonadal, the ecdysone-inducible proteins *Eip28/29* (47), and *mex1* (48).

Binding of dCREB-A to the CRE. The mammalian CREB proteins mediate transcription specifically through the CRE sequence and do not bind to the related TRE that is recognized by the AP-1 family of proteins. We wished to determine whether dCREB-A also bound only to the CRE or whether it could recognize the TRE sequence as well.

We utilized gel shift analyses to characterize the binding specificity of dCREB-A. The intact dCREB-A protein was produced in a bacterial expression system, and the amount of product was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE). One microliter of a 1:100 dilution of the extract was incubated with labeled CRE oligonucleotide, and the protein-DNA complexes were separated by nondenaturing PAGE. We found that dCREB-A was able to form a complex with the labeled CRE oligonucleotide and that the binding was specific; that is, it could be inhibited by unlabeled CRE oligonucleotide (Fig. 3A). However, whereas a 100-fold excess of unlabeled CRE completely inhibited binding of the radiolabeled probe to mammalian CREB, the binding to dCREB-A was not completely abolished. This suggests that some of the CRE binding of dCREB-A may not be specific. The TRE oligonucleotide, which differs from the CRE sequence by the deletion of a single base pair, did not compete with the labeled CRE for

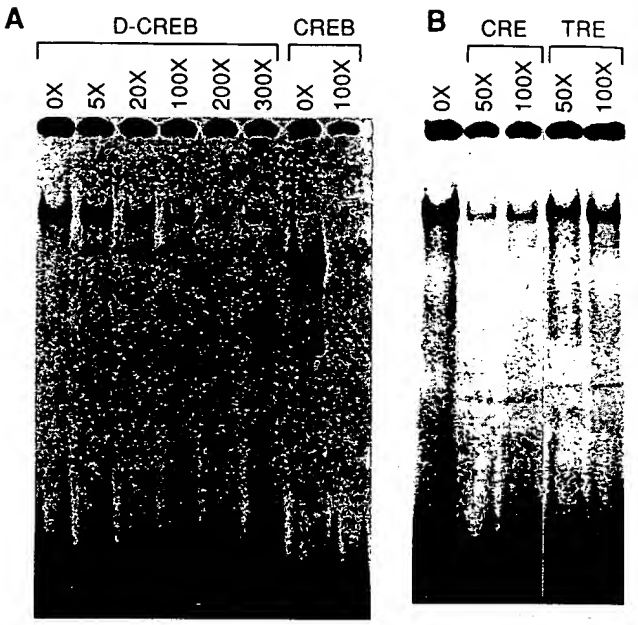

FIG. 3. Gel shift analysis of dCREB-A. (A) Protein-DNA complexes were generated as described. Each lane contains the same concentration of dCREB-A protein or, in the control lane, CREB protein and equal amounts of radiolabeled somatostatin CRE. The CRE probe was a kinased, double-stranded oligonucleotide. The lanes have increasing amounts of unlabeled somatostatin CRE added, as indicated. (B) Gels run as in panel A, but lanes have unlabeled CRE or metallothionein TRE oligonucleotides added as competitors, in the amounts indicated.

binding to either mammalian CREB or dCREB-A (Fig. 3B). We also found that CRE binding was sensitive to the concentration of Mg^{2+}. Both the mammalian and the *Drosophila* CREBs bind to the CRE in the presence of 5 mM $MgCl_2$, but only the dCREB-A protein can form complexes in the absence of Mg^{2+} ions (data not shown).

dCREB-A is a transcriptional activator in JEG-3 cells. Although the dCREB-A protein is structurally similar to the mammalian transcription factor CREB and is capable of binding specifically to the CRE sequence, it was important to determine whether this protein could activate transcription. We assayed the ability of dCREB-A to activate transcription in JEG-3 cells because they have been used extensively to characterize the activity of mammalian CREB. Because the somatostatin CRE may not be the optimal binding site for dCREB-A, we were concerned that dCREB-A might not compete well with the endogenous cAMP-responsive transcription factors for CRE binding. This problem was obviated by fusing the activation domain of the dCREB-A protein to the *Saccharomyces cerevisiae* GAL4-binding domain and using a CAT gene regulated by the GAL4 promoter as a reporter. JEG-3 cells were transfected with vectors carrying the reporter alone and with both the reporter and the dCREB-A-GAL4 fusion protein vectors. An RSV-luciferase vector was cotransfected as a control for transfection efficiency. After 24 h, half of the transfected cells were treated with cAMP and incubated for an additional 24 h. The cells were collected and assayed for luciferase and CAT activities. The results demonstrate that the dCREB-A activation domain is a strong transcriptional activator (Fig. 4). There is only a modest increase in activity in response to

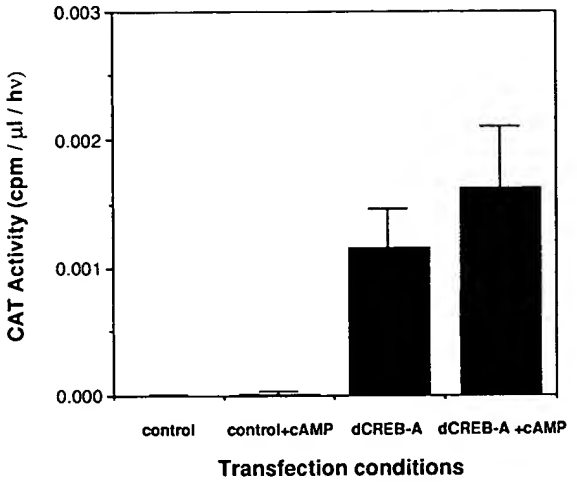

FIG. 4. Activation of transcription by dCREB-A. The dCREB-A activation domain was fused to the GAL4-binding domain, and the fusion protein, regulated by the RSV promoter, was transfected into JEG-3 cells with a GAL4-regulated CAT reporter. An RSV-luciferase vector was used as a control for transfection efficiency. The CAT activity is expressed as arbitrary units, corrected for transfection efficiency.

cAMP, consistent with the lack of consensus PKA sites in the dCREB-A protein.

Developmental expression pattern of dCREB-A RNAs. We determined the sizes of the dCREB-A transcripts and their temporal patterns of expression by using Northern (RNA) blot analyses. Total RNAs from different stages in *Drosophila* development were isolated, separated on denaturing agarose gels that were blotted onto nitrocellulose, and probed with the radioactive dCREB-A cDNA (Fig. 5). A 4.4-kb dCREB-A transcript could be detected at between 10 and 16 h of embryogenesis and later in adult heads and

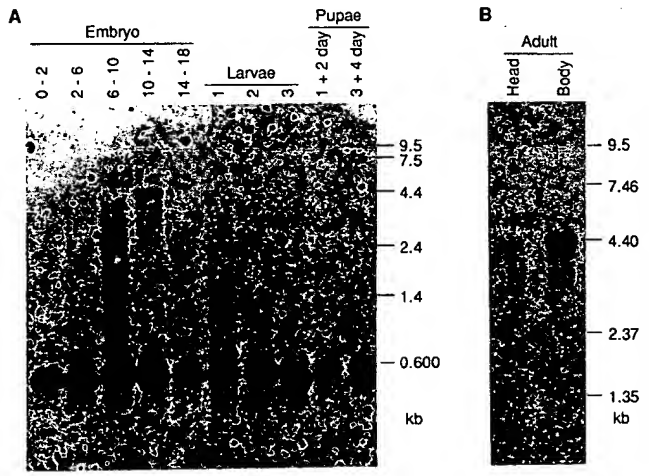

FIG. 5. Northern blot analysis of dCREB-A transcripts. RNAs from different time points in *Drosophila* development were isolated and the Northern blot was generated as described previously (45). The filter was probed with the total dCREB-A cDNA that had been labeled with [α-^{32}P]dCTP by random priming. The filters were visualized on XAR film after 5 days at −70°C with an intensifying screen. Except for the adult head and body RNAs, the RNA samples were normalized to the ribosomal protein rp49 transcript (600-bp band).

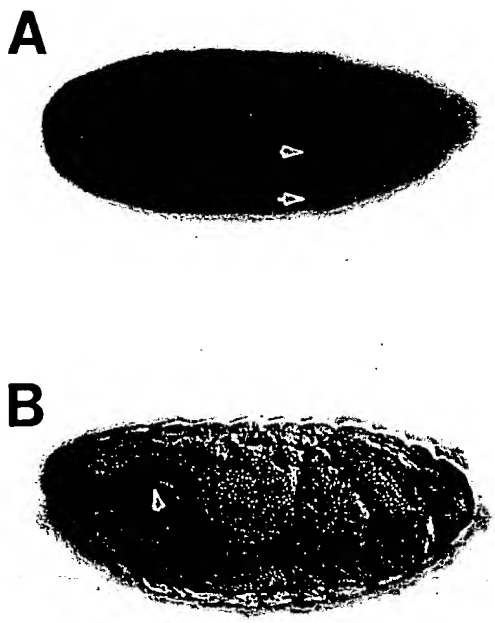

FIG. 6. dCREB-A expression pattern in embryos. Embryos were collected and hybridized with nonradioactive dCREB-A probes as described. (A) Embryo at germ band extension (after 7 to 8 h of development). dCREB-A transcripts are detected in the salivary-gland placodes (white arrows). (B) Embryo after germ band retraction after 14 to 16 h of development. Completely formed salivary glands express dCREB-A. Background staining is the same as in the control animals.

bodies. We determined the spatial pattern of dCREB-A expression by probing embryos with digoxigenin-labeled, single-stranded dCREB-A DNAs. This analysis demonstrates that the dCREB-A transcripts are first seen at germ band extension (about 7 h into embryogenesis) in the salivary-gland placodes and continue to be expressed in the salivary gland up to the 16th hour of development (Fig. 6).

dCREB-A transcription pattern in the adult. Tissue sections of adult flies were hybridized with both sense and antisense dCREB-A ^{35}S-RNA probes. Low levels of dCREB-A RNAs are detected in the cell bodies of the brain and the optic lobe, only in sections that have not been treated with RNase (Fig. 7). In the sections treated with RNase, dCREB-A RNAs are detected in the midgut epithelial cells of the cardia, the adult salivary gland, the columnar but not the squamous follicle cells in the ovary, and in the male seminal vesicle, anterior ejaculatory duct, and ejaculatory bulb. Weaker hybridization is seen in the midgut proper (Fig. 8).

DISCUSSION

dCREB-A is a member of the ATF-CREB family of transcription factors. We have described the isolation and initial characterization of a *Drosophila* CREB homolog that is capable of binding the somatostatin CRE sequence and activating transcription in cell culture. Sequence analysis of dCREB-A shows that it is structurally similar to mammalian CREB. It has a large acidic amino-terminal activation do-

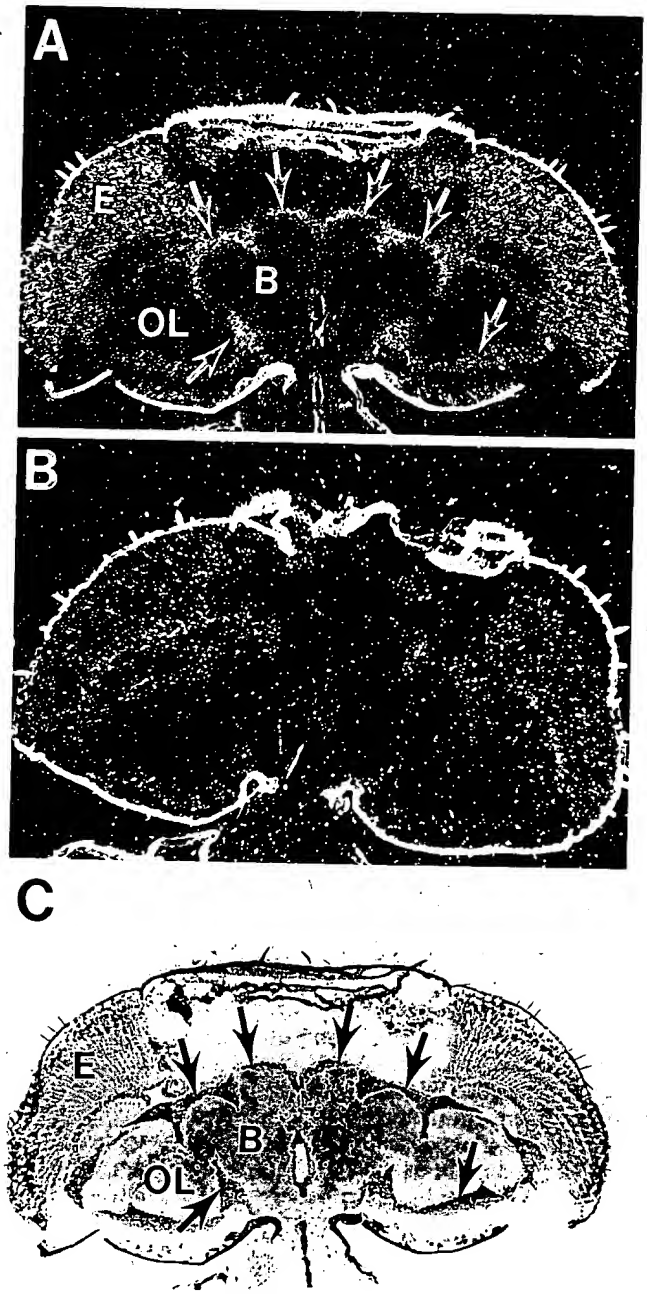

FIG. 7. dCREB-A expression pattern in adult heads. Adults were collected, sectioned, and hybridized with ^{35}S-RNA probes as described. (A) In the head, dCREB-A is expressed weakly in the cell bodies of the brain and optic lobes (arrows). (B) Except for the nonspecific binding in the eye, this hybridization is not detected in alternate head sections hybridized with sense dCREB-A probes. (C) Bright-field photomicrograph of section shown in panel A. The cell bodies of the optic lobe and brain are stained darkly (arrows). B, brain; E, eye; OL, optic lobe.

main, a highly basic DNA-binding domain, and a carboxy-terminal leucine zipper motif. Its calculated molecular mass of 56 kDa indicates that dCREB-A is somewhat larger than any of the mammalian CREB proteins.

We have categorized this protein as a member of the CREB-ATF family for several reasons. First, the DNA-binding domain of dCREB-A is 48% identical to mammalian CREB and only 23% identical to the *Drosophila* c-*jun* homolog. Second, the binding characteristics of dCREB-A are more similar to those of CREB than to those of c-*jun*. The results of the gel shift assays show that the TRE fails to compete with the CRE sequence for dCREB-A binding. Finally, we have recently shown that the dCREB-A gene, like mammalian CREB (23), contains an intron immediately upstream from the DNA-binding domain (44). The mammalian *jun* gene does not contain introns (22). Thus, the structural organization of the dCREB-A gene also supports our classification of this factor as part of the CREB-ATF family.

The 8-bp CRE core, 5′-TGACGTCA-3′, appears to account for the binding specificity of the dCREB-A protein, but as shown by the gel shift analysis, a small component of the CRE binding to this factor may be nonspecific. The *Drosophila* system should allow us to distinguish target sequences for dCREB-A from fortuitous binding sequences by a combination of genetic and molecular techniques. It is also possible that the CRE is the optimal target sequence for dCREB-A, and the specificity of binding may be increased when it forms a heterodimer with another leucine zipper protein. This phenomenon has been demonstrated for c-Myc, which exhibits an increased affinity for its binding site when complexed with a protein designated Max (6).

Unlike mammalian CREB, which contains four leucine residues within the leucine zipper domain, or the TRE-binding factors which contain five leucine residues, dCREB-A has six hydrophobic residue iterations. Helical-wheel analysis (38) of the dCREB-A zipper suggests that it, like other basic-domain leucine zipper factors, should form dimers. The work of Hu et al. (25) suggests that the tyrosine found at the third leucine position is probably a benign substitution. They have shown that a leucine-to-tyrosine mutation at the same position in the GCN4 zipper region does not affect dimerization.

The mammalian CRE-binding proteins have amino-terminal transactivation domains that are poorly conserved but frequently contain consensus phosphorylation sites for a variety of protein kinases. In this way, the CREB proteins can be utilized by different signal transduction pathways to activate CRE-containing genes. For example, mammalian CREB and ATF-1 contain phosphorylation sites for PKA and activate transcription in response to the second-messenger cAMP (16, 42). While dCREB-A is a strong transcriptional activator in cell culture, it does not stimulate additional transcription in response to cAMP. This is consistent with the fact that it does not contain a consensus PKA site.

Signal transduction pathways initiated by Ca^{2+} influx can also stimulate the expression of selected genes. For example, depolarization of neurons and the consequent elevation of intracellular calcium cause the rapid phosphorylation of cellular proteins and the activation of new transcription patterns (4, 5, 37). The element in the c-*fos* promoter that responds to calcium influx is related to the somatostatin CRE (50), and several studies have shown that CREB is probably activated through the Ca^{2+}-calmodulin-dependent kinases (51). Thus, the presence of consensus CaM kinase II phosphorylation sites within dCREB-A suggests that this factor may be activated in response to changes in membrane potential. Indeed, recent work has shown that dCREB-A is a substrate for mammalian CaM kinase II in vitro (55). However, the presence of a consensus phosphorylation site does not ensure that a factor is regulated through modification of that site in vivo. For example, CRE-BP1 can be phosphorylated by PKA but is not activated by this path-

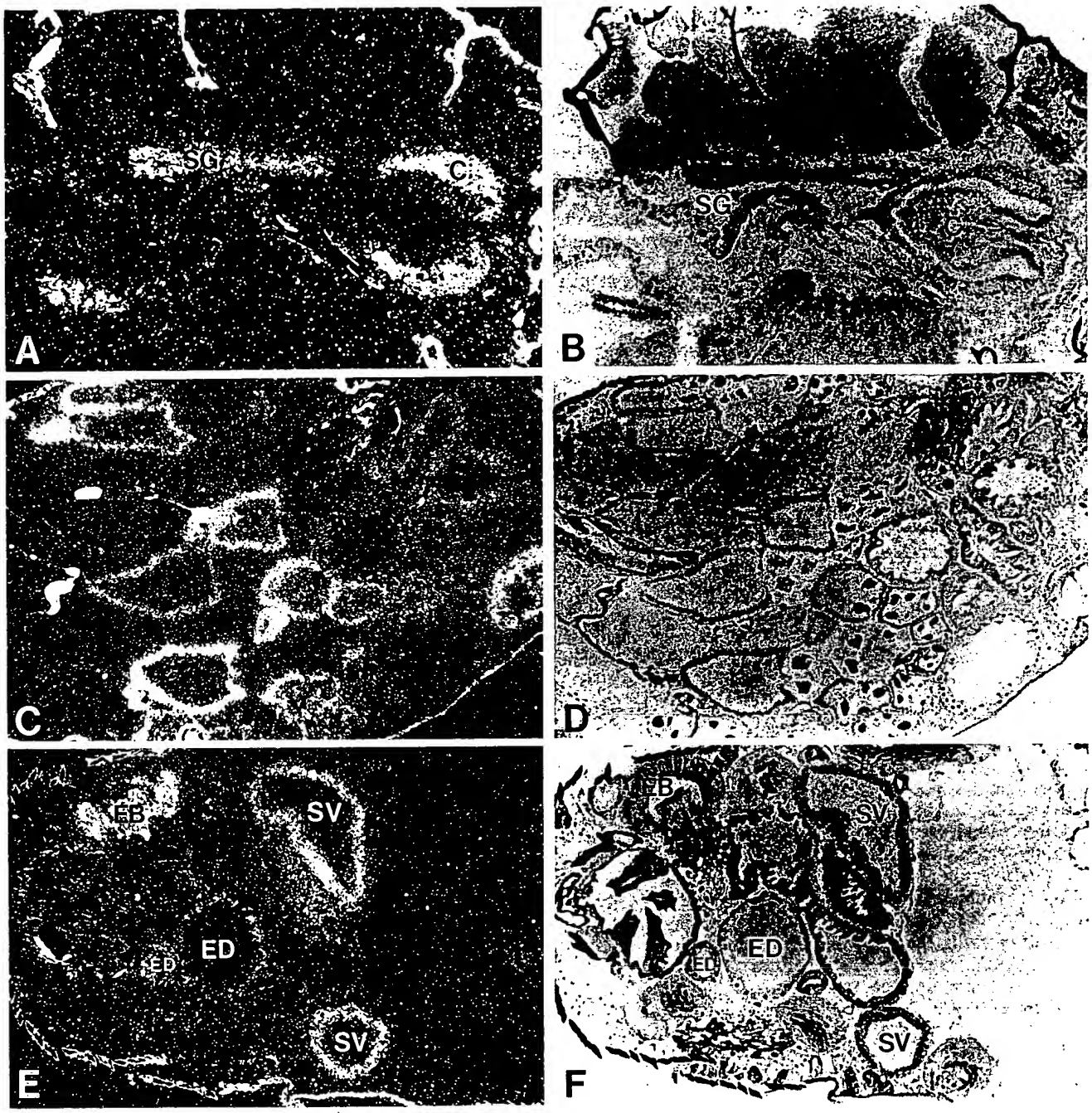

FIG. 8. dCREB-A expression pattern in adult bodies. Adults were collected, sectioned, and hybridized with ^{35}S-RNA probes as described. After hybridization, these sections were treated with RNase. The sense probe controls showed no background signal in the tissues expressing dCREB-A. Dark-field and bright-field photomicrographs showing hybridization to the midgut epithelial cells of the cardia and the salivary gland (A,B), ovarian columnar follicle cells (C,D), and the male reproductive tract (E,F). SG, salivary gland; C, cardia; EB, the ejaculatory bulb; ED, the anterior ejaculatory duct; SV, the seminal vesicle.

way. The recent cloning of *Drosophila* CaM kinase (11) may allow us to determine whether dCREB-A is a substrate for the *Drosophila* form of this enzyme.

An additional *Drosophila* CREB-like cDNA has been isolated in our laboratory (58), providing evidence that *D. melanogaster*, like mammals, contains a family of these transcription factors. At least five bZip proteins are expressed in *D. melanogaster*: c-*jun* and *fos* homologs (39), the CNC protein (34), and the two forms of *Drosophila* CREB. The mutational and molecular analysis of these proteins will determine whether they interact with each other to regulate developmental or cellular processes.

The expression pattern of dCREB-A in *Drosophila* development. The dCREB-A transcripts have a well-defined temporal and spatial pattern of expression. The dCREB-A RNAs are first detected at germ band extension in the salivary-gland placodes and continue to be expressed up to the 16th hour of development. A developmental Northern analysis

suggests that dCREB-A is not expressed in the three larval instars or pupae. However, we cannot rule out the possibility that low levels of dCREB-A RNAs are expressed in these stages and not detected in this analysis. In fact, by using an antiserum to dCREB-A, we have detected staining in the salivary-gland nuclei of the third larval instar (54). In the adult, the dCREB-A transcripts are seen in structures derived from all three germ layers. The dCREB-A RNAs are detected at low levels in the cell bodies of the brain, optic lobe, and midgut. Higher levels of expression are seen in the midgut epithelial cells of the cardia and in the salivary gland. One interesting feature of dCREB-A expression is that it has a sexually dimorphic pattern. In the female, dCREB-A RNAs are found in the ovarian columnar follicle cells that are derived from the mesoderm. In the male, dCREB-A expression is seen in structures derived from the genital disk. These results indicate that dCREB-A regulation is complex. The pattern of dCREB-A expression differs from that of dJRA, the *Drosophila* c-*jun* homolog (39, 60), which is expressed ubiquitously throughout embryogenesis. This difference suggests that dCREB-A may be restricted to regulating a limited set of developmental processes.

Without a mutational analysis of dCREB-A, it is impossible to predict the functional significance of the protein for the developing embryo and the adult fly. However, the distribution pattern of dCREB-A transcripts implies possible roles for the protein. For example, the presence of dCREB-A in the follicle cells and male reproductive tract suggests that it may be involved in fertility. The dCREB-A transcripts in the optic lobe suggest that mutations in the dCREB-A gene might result in a visual defect. Because the dCREB-A protein does not contain a PKA site, it is unlikely to be a part of the cAMP signal transduction pathway that is predicted to be involved in learning and memory by the dunce (8) and rutabaga (31) genes. However, dCREB-A expression in the brain and optic-lobe cells suggests that it may be needed for other neural functions.

Lethal ethyl methanesulfonate (EMS) mutations representing 17 complementation groups have been generated in the 71CD region (26). We have determined that four or five of these complementation groups fall in the region encoding dCREB-A (43). We are currently generating P element vectors carrying the dCREB-A gene and will use them to rescue these mutations by transformation. An analysis of dCREB-A mutants, the regulation of this gene, and the identification of target genes will certainly provide information about the role of the dCREB-A protein in *Drosophila* development. Given the evolutionary conservation of the CREB-ATF system, these studies will also provide insights into the complex pattern of CRE-mediated responses in mammals.

While the present report was under review, Abel et al. (1) described a *Drosophila* CREB-like protein, BBF-2, that is probably identical to dCREB-A. Although the single sequence difference between the two cDNAs is probably explained by a frameshift error, the cytological location of the gene and its pattern of expression are completely different. Abel et al. suggest that the BBF-2 protein plays a role in the regulation of the alcohol dehydrogenase (*ADH*) gene in the yolk and fat body. Our results do not support this conclusion. The dCREB-A transcripts are not detected in the fat body and are found in the follicle cells of the ovary but not in the egg. This distribution pattern has been confirmed by immunohistochemistry (54). We have no explanation for these discrepancies, which will no doubt be resolved by continued analysis of these two sequences.

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